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ORIGINAL ARTICLE

SIMULTANEOUS MEASUREMENT OF ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE AND GUANOSINE 3',5'-CYCLIC MONOPHOSPHATE IN BIOLOGICAL SAMPLES

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Abstract Two sensitive enzymatic fluorometric assays have been developed for adenosine 3',5'-cyclic monophosphate (cAMP) by Sugiyama et al. (Anal. Biochem. 1990) and for guanosine 3',5'-cyclic monophosphate (cGMP) by Seya et al. (Anal. Biochem. 1998). However, to make a new simultaneous comparison of two cyclic nucleotides, distinct measurement methods cause less reliability and longer measurement time. To overcome these problems, we developed a simultaneous measurement method for them. All adenosine nucleotides and GMP were enzymatically degraded using alkaline phosphatase and apyrase. The remaining GDP was converted to GTP by creatine kinase. Cyclic GMP and cAMP, absorbed into a Sep-Pak amino propyl cartridge, were eluted to separate from GTP, and then were simultaneously quantified using improved enzymatic fluorometric assay. The detection limits for cAMP and cGMP were 1 and 5 fmol, respectively. The total measurement time was about 10 h. Using this method, the basal cAMP and cGMP levels in rat aortic smooth muscle cells were confirmed to 4.1 and 0.042 pmol/mg protein, respectively. An adrenergic agonist, isoproterenol (1 μM) increased cAMP approximately 6 fold, while nitric oxide donor, S-nitroso-N-acetylpenicillamine (100 μM) increased cGMP approximately 230 fold. These results suggest that this simultaneous measurement of cGMP and cAMP can provide a more convenient assessment of them in biological samples.

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Key words: adenosine 3',5'-cyclic monophosphate; guanosine 3',5'-cyclic monophosphate; Sep-Pak amino propyl cartridge; enzymatic cycling; aortic smooth muscle cells.

Introduction

Intracellular adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP) are typical second messengers, starting intracellular signaling cascade[1]. Generally, cAMP- and cGMP-dependent intracellular signaling pathways produce opposite effects on various functions of cardiovascular system, central nervous system and other functions such as the sugar uptake system[2-4]. Namely, cAMP stimulated cardiac positive inotropic and chronotropic functions, while cGMP decreased them[5]. It is very important to accurately assess the intracellular behavior of both cAMP and cGMP in various tissues.

To assay the levels of intracellular cyclic nucleotides, Sugiyama *et al.*[6] and Seya *et al.*[7-9] developed a sensitive assay for cAMP and cGMP, respectively, consisting entirely of enzymatic reactions. Their assay methods achieved high sensitivity using enzymatic cycling which effectively amplifies a desired substance. These methods have two advantages, *i.e.*, shorter measurement time and highly sensitive linear calibration curve, compared with traditional enzymatic immunoassay[10]. However, for simultaneous measurement of intracellular or tissue cAMP and cGMP using these methods, these advantages are diminished because their distinct pretreatment methods trigger not only longer measurement time but also less reliable

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comparison between each cyclic nucleotide level. To solve this problem, it is necessary to develop a common convenient method for isolation of cAMP and cGMP.

In the present study, we combined the enzymatic reaction and the filtration method using a weak anion exchanged cartridge resin to develop a convenient method to isolate cAMP and cGMP, simultaneously. Using this method, we could accurately measure the basal cAMP and cGMP levels in the rat aortic smooth muscle cells and easily assess slight changes of the intracellular cAMP and cGMP levels induced by various stimulations.

Materials and Methods

Biochemicals

The enzymes and substrates used in this study, phosphodiesterase 3', 5'-cyclic nucleotide specific guanylate kinase, lactate dehydrogenase, nicotinamide adenine dinucleotide (reduced form), phospho(*enol*)pyruvate, pyruvate kinase, succinic thiokinase, adenine, and guanine nucleotides, were obtained from Sigma-Aldrich Co. (St. Louis, MS, USA), and the remaining biochemicals were obtained from Wako Chemical Co. (Osaka). Sep-Pak cartridges were purchased from Waters Co. (Milford, MA, USA). Measurement of cAMP and cGMP was performed in triplicate.

For culture experiments, Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo). L-Glutamine was purchased from Nacalai Tesque, Inc. (Kyoto). Fetal bovine serum (FBS) and Penicillin & Streptomycin were obtained from Nichirei Co. (Tokyo) and Life Technologies Inc. (Burlington, ON, Canada), respectively. All chemicals used were of the highest purity commercially available. All solutions were made fresh at sufficiently high concentrations so that only very small aliquots had to be added to the culture medium.

Male Wistar rats at 10 weeks of age were purchased from CLEA Japan, Inc. (Tokyo). This study was performed in accordance with Guidelines for Animal Experimentation, Hirosaki University and was approved by the Animal Care and Use Committee. The animals were maintained for 1 week at a temperature of 22 ± 2℃ and relative humidity of 57% on a 12-h light / 12-h dark (lights on 08:00 – 20:00) schedule and had free access to water and food.

Simultaneously measurement of cAMP and cGMP

This method consists of two procedures: 1) the first procedure for isolation of both cyclic nucleotides from other nucleosides and nucleotides, and 2) measurement of cAMP and cGMP (Figure 1).

1. Isolation of cAMP and cGMP

Step 1: Degradation of AMP, ADP, ATP and GTP.

Ten μL of neutralized tissue or cell extract or 10 μL of known amounts of cAMP and cGMP as an authentic standard containing 20 mM Tris–HCl buffer adjusted to pH 8.0 was added into a polypropylene tube. Ten μL of reaction mixture containing 20 mM Tris–HCl buffer adjusted to pH 8.0, 2 mM $MgCl_2$, 4.0 units/mL apyrase, 20 units/mL adenosine deaminase, and 40 units/mL alkaline phosphatase was added to each tube. The mixture was incubated at 30°C for 1 h. After incubation, enzymes were degenerated by heating at 90°C for 10 min.

Step 2: Phosphorylation of GDP to GTP

Ten μL of reaction mixture containing 20 mM Tris–HCl buffer adjusted to pH 8.0, 2 mM $MgCl_2$, 1 mM creatine phosphate, and 80 units/mL creatine kinase was added to each tube. The mixture was incubated at 30°C for 1 h. After incubation, enzymes were degenerated by heating at 90 °C for 5 min.

Step 3: Isolation of cAMP and cGMP.

Purified water (470 μL) was added to the reaction mixture, and the mixture (final volume

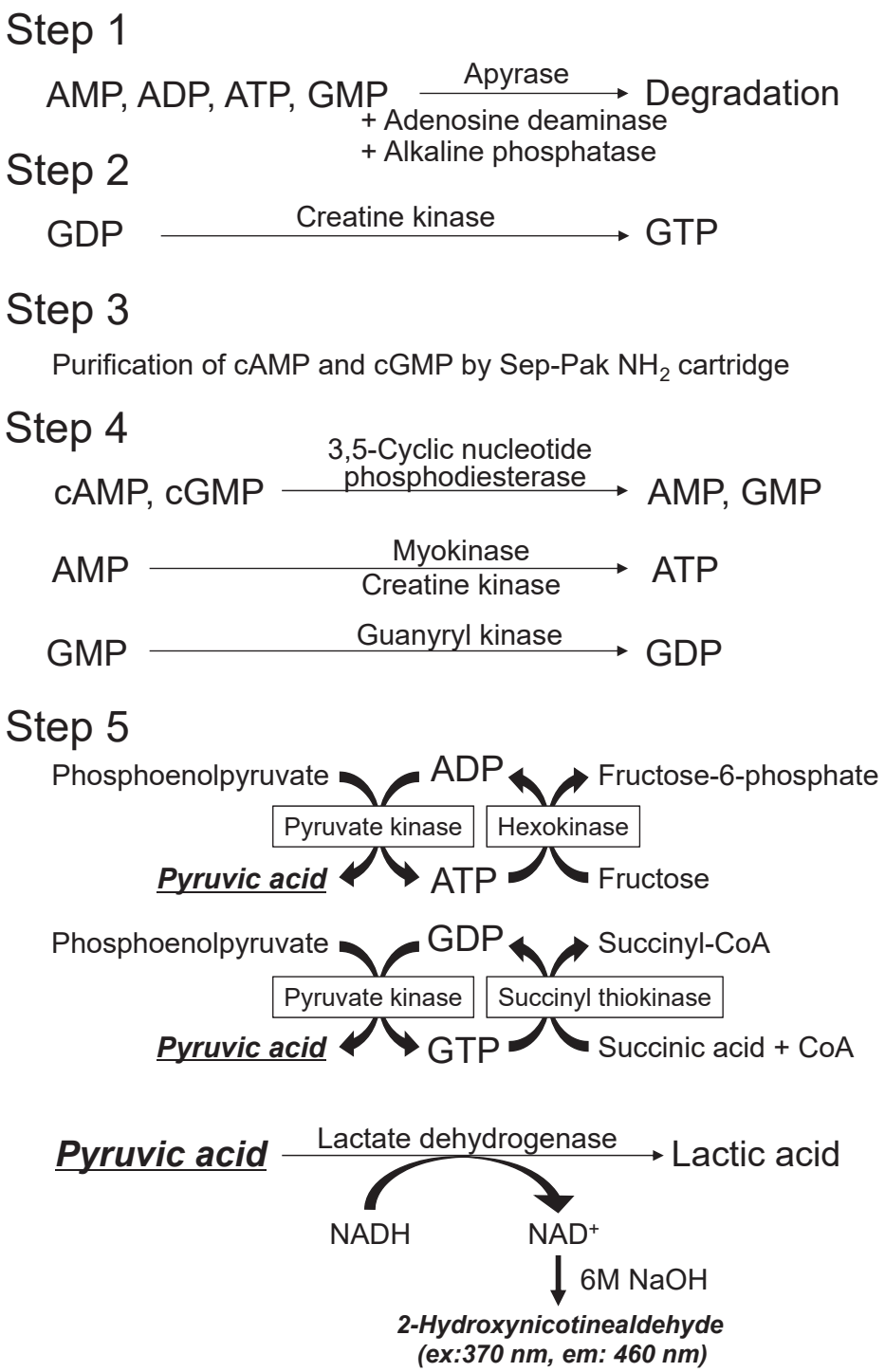

Figure 1 Schemes about the method to isolate cAMP and cGMP (Steps 1-3) and the principle of the fluorometric assay for cAMP and cGMP (Steps 4-5).

was 500 μL) was absorbed into a Sep-Pak amino propyl cartridge containing weak anion exchanged resin (500 mg). After the cartridge was washed with 500 μL of purified water, cAMP and cGMP absorbed in this cartridge were eluted with 3 mL of purified water. The eluent including cAMP and cGMP was concentrated under reduced pressure at 60°C

for 2 h. A set of triplicate samples was similarly assayed and used for an internal tissue blank control. The cAMP or cGMP concentrated in each sample was diluted with 20 mM Tris-HCl adjusted to pH 8.0 (40 μL).

2. Measurement of isolated cAMP and cGMP

Step 4: Ring cleavage reaction followed by phosphorylation of cAMP and cGMP

Two and 8 μL of reaction mixture for measuring cAMP and cGMP, respectively, were added into distinct 75-mm fluorometer tubes. We also prepared known amounts of cAMP and cGMP containing 20 mM Tris–HCl buffer adjusted to pH 7.5 as a standard. We further added 5 μL of a reaction mixture containing 20 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$, 100 μM $CaCl_2$, 300 unit/mL calmodulin, and 0.1 unit/mL 3', 5'-cyclic nucleotide specific phosphodiesterase to each tube. A blank was generated by adding buffer (5 μL) containing 20 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$, 100 μM $CaCl_2$, and 300 unit/mL calmodulin to the parallel set of samples. After 1 h of incubation at 30°C, the reaction mixture was provided for the next step.

To synthesize cAMP-derived ATP, a reaction mixture (5 μL) containing 20 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 100 mM KCl, 2 nM ATP, 100 μM creatine phosphate, 500 μM dithiothreitol, 120 units/mL myokinase and 40 units/mL creatine kinase was added to the assay tubes from Step 4. The reaction mixture was incubated for 1 h at 30°C. After incubation, enzymes were degenerated by heating at 90°C for 5 min.

On the other hand, to synthesize cGMP-derived GDP, same reaction mixture (5 μL) containing 20 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 100 mM KCl, 50 μM ATP and 1 unit/mL guanylate kinase was added to the assay tubes from Step 2. The reaction mixture was incubated for 1 h at 30°C. After incubation, enzymes were degenerated by heating at 90°C for 5 min.

Step 5: Enzymatic cycling reaction followed by fluorescence measurement of amplified pyruvic acid

ATP and GDP preparation followed by enzymatic cycling reaction from cAMP-derived AMP and cGMP-derived GMP, respectively, were performed as described previously[6, 7].

Amplified pyruvic acid level produced by each enzymatic cycling reaction of cAMP-derived ATP and cGMP-derived GDP was measured by fluorometric assay. Namely, we added 100 mM Tris–HCl (pH 8.0), 5 mM NADH, and 4 units/mL lactate dehydrogenase to each assay tube containing 10 μL of reaction mixture. After 10 min reaction at room temperature, 10 μL of 2M HCl was added to the reaction mixture, destroying excess NADH after 10 min at room temperature. Finally, 1 mL of 6M sodium hydroxide was added to the reaction mixture, which was warmed at 60°C for 10 min. After cooling the reaction mixture to room temperature, the fluorescent intensity of the reaction mixture was measured at 370 nm as the excited wavelength and 460 nm as the emitted fluorescence wavelength[11].

Cell Culture

After anesthetizing rats (10 weeks of age), thorax was opened and the descending thoracic aorta was carefully removed, cleaned of adhering fat and connective tissue. The endothelium of aorta was denuded gently by rubbing the intima surface of aorta with glass rod. Aortic smooth muscle cells were obtained by collagenase digestion of endothelium-denuded aorta, and were cultured as described by Kanemaru et al.[12]. The culture medium used was DMEM containing L-glutamine (584 mg/L), 10% FBS, 10 U/L penicillin, and 10 μg/L streptomycin. Cells from passages 3 to 5 were used for all experiments. The cells were cultured at 37°C under 5% CO_2, and the

medium was replaced with fresh medium every 3 days.

Measurement of cAMP and cGMP in cells

When the cultured rat smooth muscle cells reached 90% confluence in 35 mm dish, the medium was exchanged by tyrode solution including 200 μM 3-isobutyl-1-methylxanthine (IBMX), a nonselective phosphodiesterase inhibitor, and then incubated for 15 min. After 5 min of administration of various reagents at 37°C, the medium was exchanged to cold 10% trichloroacetic acid solution (500 μL) and let to stand at room temperature for 30 min. The supernatant was washed with water-saturated ether four times (5 mL). After concentrating washed solution, residue was collected for cAMP and cGMP determination using our improved enzymatic assay described as above. Protein content was determined by the Bradford method[13].

Statistical Methods

Multiple group comparisons were performed by one-way ANOVA, followed by Schéffe procedure for comparing means. Values are presented as means ± SD. $P < 0.05$ was considered to be statistically significant.

Results

Simultaneous Isolation of cAMP and cGMP from Biological Samples

Figure 1 depicts the steps for the isolation of cAMP and cGMP. In step 1, apyrase and alkaline phosphatase dephosphorylate and degenerate AMP, ADP, ATP and GMP but not GDP and GTP. In this reaction, adenosine produced was further degraded by adenosine deaminase. In steps 2, the remaining GDP was converted into GTP using creatine kinase and excess creatine phosphate, and then in step 3, cAMP and cGMP were simultaneously separated from GTP using Sep-Pak amino propyl cartridge column.

Figure 2 depicts the elution profile of cAMP (200 fmol), cGMP (200 fmol) and GTP (10 nmol) from a Sep-Pak cartridge in step 3, which was composed of weak anion exchanged resin. We used purified water as an eluent. Previous elution with 500 μL could remove degraded compounds. GTP bound to the column was not eluted with the next 3 mL of water, but cAMP and cGMP were easily eluted.

During these isolation steps, at least 10 nmol of AMP, ADP, GMP, GDP, and GTP, and 100 nmol of ATP were completely removable. However, larger volume of GTP (50 nmol) was leaked a little from cartridge in step 3 as 6.0 ± 2.8 pmol ($n = 4$) causing very high background.

Calibration Curve

Figure 1 depicts the measurement of isolated cAMP and cGMP. Isolated cAMP was converted to ATP through the ester cleavage reaction by phosphodiesterase and then the phosphorylation reaction in the presence of myokinase and creatine kinase. To amplify the production of pyruvic acid, we applied hexokinase and pyruvate kinase to the enzymatic cycling[7]. On the other hand, isolated cGMP was converted to GDP by phosphodiesterase followed by guanylate kinase. To amplify the production of pyruvic acid, we applied succinic thiokinase and pyruvate kinase to the enzymatic cycling[10]. The addition of 10 μL of 1M EDTA ended the enzymatic cycling.

Pyruvic acid amplified by enzymatic cycling of each cGMP-derived GDP and cAMP-derived ATP was reduced to lactic acid by lactate dehydrogenase along with the oxidation of NADH to produce NAD^+. Excess NADH was degraded by 10 mL of 2M HCl. Finally, NAD^+ was converted to 2-hydroxynicotinealdehyde by 6M sodium hydroxide at 60°C for 10 min. The produced 2-Hydroxynicotinealdehyde was excited at 370 nm and emitted fluorescence at

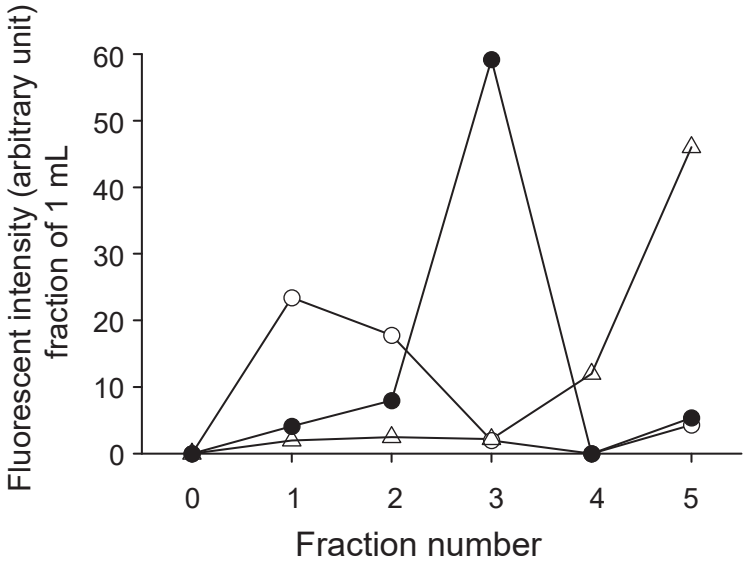

Figure 2 Typical elution profiles of cAMP, cGMP and GTP on the SEP-PAK amino propyl cartridge. An aliquot of each fraction was subjected to a fluorescent assay. The fluorescent intensity of 2-hydroxynicotinealdehyde was measured with the level corresponded to the amount of amplified cAMP, cGMP or GTP. Two hundred femtomoles of cAMP (●) and cGMP (○) were sufficiently separated from 10 nmol GTP (△) by 3 mL elution of purified water through the cartridge.

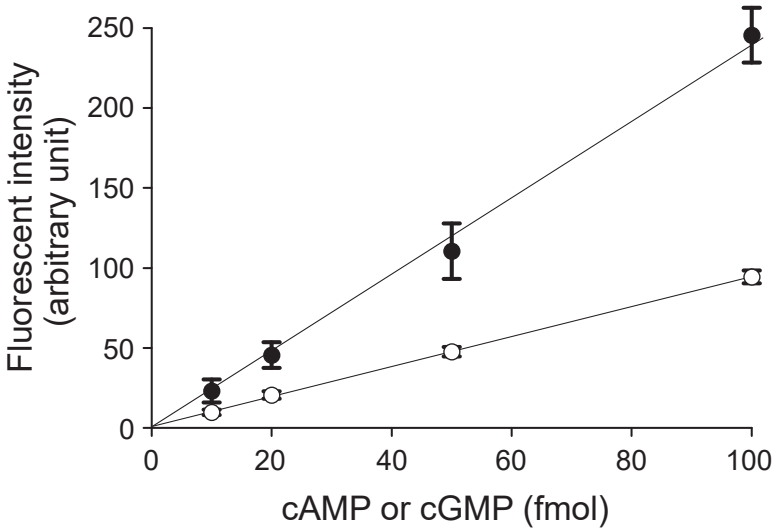

Figure 3 Calibration curves of cAMP (●) and cGMP (○). The enzymatic cycling reaction lasted for 2 hours. The fluorescent intensity of 2-hydroxynicotinealdehyde corresponding to the amount of amplified cAMP and cGMP was measured. Each value is the mean ± SD of seven experiments.

460 nm in strong alkali[11].

Calibration curves for cAMP and cGMP were estimated over the reaction time range of 2 h for the enzymatic cycling (Figure 3). Sufficient linearity treated by linear regression analysis was observed in the range up to 100 fmol of each cAMP and cGMP by 2 h of the enzymatic cycling. When 20 nmol NADH was used for this assay, the calibration curve of both cAMP and cGMP sufficiently became linear up

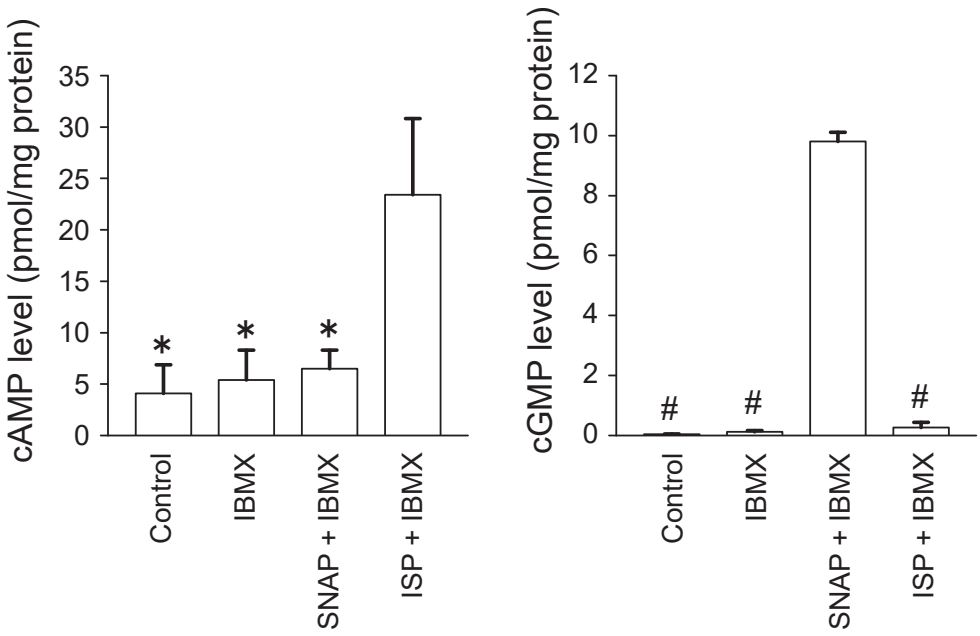

Figure 4 Changes in the intracellular cAMP and cGMP levels in the aortic smooth muscle cells in the presence of various drugs. Control, basal level; IBMX, in the presence of 3-isobutyl-1-methylxanthine (IBMX, 200 μM); SNAP + IBMX, in the presence of S-nitroso-N-acetylpenicillamine (SNAP, 100 μM) and IBMX (200 μM); ISP+IBMX, in the presence of isoproterenol (ISP, 1 μM) and IBMX (200 μM). Each value is the mean ± SD of five experiments. $^{*}p < 0.01$ vs ISP + IBMX. $^{\#}p < 0.001$ vs SNAP+ IBMX.

to 4 pmol. When the enzymatic cycling started from Step 4 was performed over 2 h, the cycle number of the enzymatic cycling of cAMP-derived ATP and cGMP-derived GDP reached to approximately 4000 and 2000, respectively. The detection limit for cAMP was 1 fmol and for cGMP was 5 fmol (signal-to-noise ratio is up to 3), respectively. The recovery ratios of cAMP and cGMP compared with non-treated authentic ones were 92.5 ± 8.0 and 89.6 ± 5.1% (n = 7), respectively.

cAMP and cGMP Levels in Smooth Muscle Cells Extracted from Rat Thoracic Aorta

As shown in Figure 4, the basal cAMP and cGMP level in cultured rat aortic smooth muscle cells were 4.1 ± 2.8 pmol/mg protein and 42.4 ± 12.1 fmol/mg protein (n=5), respectively. Isoproterenol, a β-adrenergic receptor agonist, in the presence of 3-isobutyl-1-methylxanthine (IBMX) further increased cAMP level to 23.4 ± 7.4 pmol/mg protein (n = 5, $p < 0.01$ vs IBMX alone) but not cGMP (268.2 ± 175.6 fmol/mg protein). On the other hand, S-nitroso-N-acetylpenicillamine, a nitric oxide donor, in the presence of IBMX significantly increased cGMP level to 9.8 ± 3.0 pmol/mg protein (n = 5, $p <$ 0.001 vs IBMX alone) but not cAMP level (6.5 ± 1.8 pmol/mg protein). IBMX alone did not affect the kinetics of cAMP and cGMP.

Discussion

We developed a simultaneous measurement method for cAMP and cGMP incorporating the filtration through weak anion-exchange resin. The detection limit of this assay was 1 fmol for cAMP and 5 fmol for cGMP. Excellent linearity for cAMP and cGMP, respectively, was obtained in a range up to 100 fmol, when the enzymatic cycling was performed over 2 h. The time required for measuring cAMP and cGMP using

this novel assay method is approximately 10 h, including 2 h of enzymatic cycling after removal of protein from biological samples. The most sensitive and popular radioimmunoassay for cAMP and cGMP takes 2 days due to its long acetylation reaction[14]. These facts suggest that this novel sensitive method is more convenient than other methods and can measure cAMP and cGMP within a day.

We applied the Sep-Pak cartridge to remove GTP and other degraded compounds except cAMP and cGMP in this assay. This cartridge cannot completely absorb a large amount of GTP (50 nmol) due to a little leakage of GTP and high background of fluorescence intensity. However, this amount does not affect the measurement of cAMP and cGMP, because the wet weight of any biological tissues we used is mainly less than 10 mg which contains less than 10 nmol of GTP. Furthermore, biological tissues (heart, muscle, liver, etc) include GTP less than 8 nmol/mg protein[15]. From these reasons, purified water is the best eluent as shown in Figure 2. If the Sep-Pak cartridge we used can trap both GTP and ATP, we could develop a more convenient method, namely, one step enzymatic transformation of adenosine and guanosine nucleotides except cAMP and cGMP to GTP and ATP. Unfortunately, this cartridge only traps up to 1 nmol of ATP due to high background of fluorescence intensity. The development of materials which can absorb more than 1 nmol of ATP will contribute to the improvement of this method.

The cycling enzymes, hexokinase/pyruvate kinase for ADP and/or ATP and succinic thiokinase/pyruvate kinase for GTP and/or GDP, are well known reagents for amplifying[6, 7]. Their amplification activity of ATP/ADP cycling is almost two times larger than that of GTP/GDP cycling (Figure 3). Succinic thiokinase also recognizes IDP (inosine diphosphate) and/or ITP (inosine triphosphate) as substrates for enzymatic cycling due to the high background. However, the effect of IDP and ITP in our method can be ignored because they are easily degenerated by apyrase during isolation step.

The detection limit for cAMP is 1 fmol in this improved method, which is same as in the method developed by Sugiyama et al.[6]. On the other hand, the detection limit for cGMP is 5 fmol, maintaining previous method developed by Seya et al.[7], but it is obviously lower than that in the enzyme immunoassay and radioimmunoassay[13]. However, the excellent linear calibration curve for cAMP and cGMP in our improved assay can more easily and accurately read cAMP and cGMP levels in biological samples. One limitation of the present study is the insufficient and dispersed recovery rate of both cAMP and cGMP in our developed simultaneous measurement method. To improve this limitation, it is necessary to develop a newly weak anion cartridge column having an ability to separate cAMP and cGMP more accurately than Sep-Pak amino propyl cartridge column.

To prove the advantage of this method, we measured the alteration of intracellular cAMP and cGMP in cultured rat thoracic aorta smooth muscle cells. The basal level of cAMP and cGMP measured by traditional methods has been variously reported from 0.7 to 21 and from 0.08 to 0.7 pmol/mg protein, respectively[16-20]. Using linear calibration curves, we can clearly calculate the basal levels of cAMP and cGMP as 4.1 pmol/mg protein and 42.4 fmol/mg protein, respectively. In this study, isoproterenol, a β-adrenergic receptor agonist, and S-nitroso-N-acetylpenicillamine, a nitric oxide donor, strongly raised cAMP and cGMP levels, respectively. In this experiment, we used IBMX to prevent the degradation of cAMP and cGMP by 3', 5'-cyclic nucleotide phosphodiesterase. IBMX alone did not affect both basal level of cAMP and cGMP. Those large changes mean that our improved method can easily provide detailed information

such as slight alterations of intracellular cAMP and cGMP caused by other activators and inhibitors on various intracellular signaling pathways. We have previously confirmed that the basal level of cGMP during electrical driving at 1 Hz (29.0 fmol/mg protein) in rat cardiac atrial strip preparation and this value is similar to the result of the previous study[7]. These results show that this improved method is useful and highly sensitive and can be applied to various biological samples for simultaneous and accurate measurement of cAMP and cGMP.

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